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The *Arabidopsis HY2* Gene Acts as a Positive Regulator of NaCl Signaling during Seed Germination

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Abstract: Phytochromobilin (PΦB) participates in the regulation of plant growth and development as an important synthetase of photoreceptor phytochromes (phy). And *Arabidopsis* Long Hypocotyl 2 (*HY2*) appropriately works as a key PΦB synthetase. However, whether *HY2* takes part in plant stress response signal network remains unknown. Here, we described the function of the *HY2* in NaCl signaling. The *hy2* mutant was NaCl-insensitive, whereas *HY2*-overexpressing lines showed NaCl-hypersensitive phenotypes during seed germination. The exogenous NaCl induced the transcription and the protein level of *HY2* which positively mediated the expression of downstream stress-related genes of *RD29A*, *RD29B* and *DREB2A*. Further quantitative proteomics showed the patterns of 7,391 proteins under salt stress. HY2 was then found to specifically regulate 215 differentially regulated proteins (DRPs) which, according to GO enrichment analysis, were mainly involved in ion homeostasis, flavonoid biosynthetic & metabolic, hormone response (SA, JA, ABA, ethylene), reactive oxygen species (ROS) metabolic, photosynthesis and detoxification pathway to respond to salt stress. More importantly, ANNAT1-ANNAT2-ANNAT3-ANNAT4 and GSTU19-GSTF10-RPL5A-RPL5B-AT2G32060, two protein interaction networks specifically-regulated by HY2, jointly participated in the salt stress response. These results direct the pathway of *HY2* participating in salt stress, and provide new insights for the plant to resist salt stress.

Keywords: *Arabidopsis*; *HY2*; salt stress; seed germination; proteome; DRPs

1. Introduction

Saline soil is an unfavorable environmental factor that seriously affects seed germination, seedling growth and even final yield in crops [1-3]. About 7% of the total land surface and 20% of the irrigated land are affected by soil with excessive salt concentration, and the situation is getting worse [4,5]. Global climate change and poor irrigation water quality are the main factors leading to soil salinization [6,7]. Therefore, it is urgent to study the molecular mechanism of plants' adaption

to saline soil and the enhancement of such adaptability of plants to saline soil through molecular genetic improvement.

As salt stress gives rise to ion stress, osmotic stress, secondary stress and oxidative stress [8,9], it is crucial for plants to maintain the balance between ion, osmosis and ROS. And plants have evolved a series of mechanisms to maintain salt balance in the long process of evolution [10]. In terms of ionic stress, after the perception of salt-stress signal induced by high concentrations of salt in plants, the salt receptor glycosyl inositol phosphorylceramide (GIPC) [11,12] can directly bind to the external Na^+ to form an direct interaction which activates the Ca^{2+} channel. The influx of Ca^{2+} is thus caused to drive the adaptive response to high salt levels, which promotes EF-hand Ca^{2+} binding proteins SOS3 to activate serine/threonine protein kinase SOS2, and then to activate Na^+/H^+ antiporter SOS1 to pump Na^+ out of the cell, thus maintaining the salinity balance in vivo [13,14]. In terms of osmotic stress, the synthesis of compatible osmolytes is crucial to maintain osmotic potential and protein structure in cells, including the expression of related genes as PM-located protein-OSCA, MAPK cascades, SnRK2 isoforms, etc. [15-17] and the synthesis of accumulation of related substances as proline, betaine, sugars, etc. [18-20]. In terms of oxidative stress, both the gene expressions as MAPKKK-MAPKK-MAPK (mitogen-activated protein kinase) cascade [21] and the ROS scavenging enzymes as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), etc. [22] act to maintain the balance of ROS in vivo. So improving the molecular mechanism of salt stress is very important to the strengthening of salt tolerance in plants.

PΦB is an open-chain tetrapyrrole chromophore, which is a critical synthetase for phytochromes to function as a light receptor to regulate plant growth and development [23,24]. *Arabidopsis HY2* encodes an key synthase of PΦB [25,26], which is a ferredoxin-dependent biliverdin reductase that catalyzes the reduction of the A-ring 2,3,3^1,3^2-diene system to produce an ethylidene group for assembly with apophytochromes [27]. Furthermore, it has been reported that *HY2* induces the synthesis of *phyA* to inhibit the elongation of hypocotyl under the far-red light treatment [28]. Under the treatment of exogenous trehalose, the expression of *HY2* is up-regulated by 2.8 times [29]. Besides, *HY2* participates in the apoplastic and chloroplastic redox signaling networks, being responsible for chlorophyll biosynthesis [30]. However, whether *HY2* is involved in plant stress response signal network remains unknown.

In this study, we found that that *Arabidopsis* PΦB synthetase *HY2* is a positive regulator in NaCl signaling during seed germination. The knockout of *HY2* enhanced plant NaCl insensitivity during seed germination, and *HY2*-overexpressing lines showed NaCl-hypersensitive phenotypes. QPCR analysis and luciferase assay also showed that exogenous NaCl could induce the expression of *HY2*. Meanwhile, we conducted Tandem mass tags (TMT)-based proteomics analysis [31] to compare col4 (wide type) and *hy2* mutant under salt stress to identify salt stress inducing HY2-specific responsive proteins. This would help to demonstrate the role of *HY2* in salt stress response pathway. We identified 9,203 proteins of col4 and *hy2* mutant in total. Moreover, HY2 is found to specifically regulate 215 DRPs, which, according to GO enrichment analysis, are mainly involved ion homeostasis, flavonoid biosynthetic & metabolic, hormone response (SA, JA, ABA, ethylene), reactive oxygen species (ROS) metabolic, photosynthesis, defense response and detoxification pathway to respond to salt stress. Our study actually reveals the plant salt stress response and identifies new elements in salt stress pathway, which provides new insights into genetic engineering of the crops to improve salt tolerance and yield.

2. Results

2.1. Disruption of *HY2* reduces, and overexpression of *HY2* enhances, NaCl sensitivity during seed germination

To analyse the novel salt tolerance genes, we used luciferase reporter system to construct different *Arabidopsis* transgene lines overexpressing firefly luciferase (LUC). Comparing with col4 and *LUC-Vector* lines, we observed that the lines expressing the PΦB synthetase HY2 showed a salt-hypersensitive phenotype and *hy2* mutant displayed a salt-resistant phenotype with 200 mM NaCl stress (**Figure 1A**). Gene lines overexpressing *HY2* and *hy2* mutant were then used to study the physiological function of *HY2* in seed germination (**Figure S1**). We found most *hy2* mutant seedlings germinated 3 days after being sown in the medium containing 200 mM NaCl, while the seedlings of col4 and *LUC-Vector* needed 4-5 days to germinate; comparing with col4, the germination rate of lines overexpressing *LUC-HY2* was significantly lower, but that of *hy2* mutant was obviously higher (**Figure 1B**).

The following QPCR analysis showed that with 0 h and 1 h NaCl stress, the transcription level of *HY2* were undifferentiated; with 3 h and 5 h NaCl stress, the transcription level of *HY2* increased by 1.7 and 2.8 times, respectively **(Figure 1C).** The luciferase assay showed a 1.5-fold increase in the protein level of *HY2* after 3 h NaCl stress (**Figure 1D**). These results indicated that NaCl significantly mediates and up regulates the *HY2* both on its transcription level and protein level. At the same time, we analyzed the expression of *HY2* in different tissues, showing that *HY2* was expressed in different tissues of *Arabidopsis*, but its expression level was the lowest in root and the highest in flower **(Figure 1E)**.

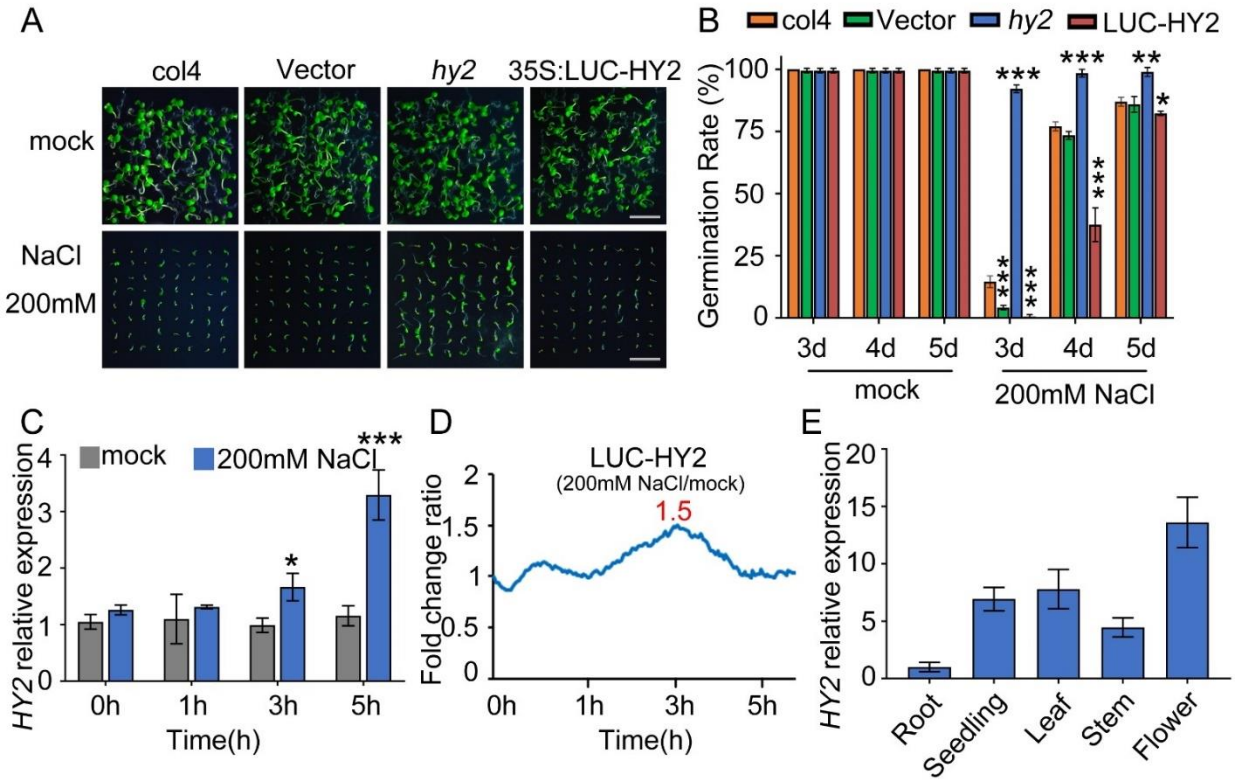


Figure 1. ABA-sensitivity of *hy2* mutant and *HY2*-overexpressing lines. (A) Phenotypic comparison. Col4, *LUC-vector*, *hy2* mutant and *LUC-HY2* overexpression lines were sown respectively on 1/2 MS medium (as mock) or 1/2 MS medium containing indicated concentration of 200 mM NaCl for 4 d. Scare bar: 1cm. **(B)** Seed germination assay. Seeds were transferred

to 1/2 MS containing 200 mM NaCl, and then the seed germination rates were calculated at 3-5 d. Data are shown as mean ± SD (n = 3). More than 64 seeds were measured in each replicate. **(C)** QPCR analysis of *HY2* expression in 5 d-old col4 seedlings treated with or without 200 mM NaCl for 0-5 h. Data are shown as mean ± SD (n = 3). **(D)** LUC signals in 5 d-old *LUC-HY2* overexpression line seedlings treated with or without 200 mM NaCl. Signals were detected in every 10 min, the detecting period is 5 h. **(E)** QPCR analysis of *HY2* expression in different tissues of *Arabidopsis*. Data are shown as mean ± SD (n = 3).

2.2. Quantitative proteomics analysis of col4 and *hy2* mutant under salt stress

To identify the mechanism of *HY2* to NaCl response pathway, we treated col4 and *hy2* mutant with NaCl stress, and then used TMT-based proteomics to figure out how HY2 protein specifically regulates the expression of salt stress-related proteins at the protein level (**Figure 2A,Table S1**). In our experiment, a total of 81,898 peptides and 68,002 unique peptides were matched with the *Arabidopsis* library; 9,203 proteins were identified and 7,391 proteins were quantified (**Figure S2A)**. The size of most identified proteins was in the range of 20-80 kDa, accounting for 74% of the identified proteins (**Figure S2B**). The distribution of peptides indicated that the amount of the corresponding proteins decreased with the increase of the peptide number **(Figure S2C)**. The protein sequence coverage of 0-10%, 5-10%，10-20%, 20-30%, 30-40%, 40-50%, 50-60% and > 60% were censused as 15%, 14.2%, 21.9%, 16.6%, 12.6%, 9.3%, 5.8%, 4.6%, respectively (**Figure S2D**). The principal component analysis (PCA) showed that the contribution ratio of principal component PC1 and PC2 were 54.0% and 21.3% respectively, and the results showed an identical repeatability of the same experimental group. More interestingly, the expression level of proteins in col4 and *hy2* mutant significantly varied under NaCl stress, indicating that HY2 protein specifically regulates protein expression concerning NaCl response pathhway (**Figure 2B, Figure S3A**). And the heatmap of the expression level of all proteins showed a different protein expression pattern in col4 and *hy2* mutant (**Figure S3B**). For a further verification of the repeatability of the experiment and an identification of the protein expression level difference between col4 and *hy2* mutant, we conducted pearson correlation coefficient of the overall expression level, with a result that the correlation of repeatability was more than 0.9, but the correlation of protein expression level in col4 and *hy2* mutant was about 0.5 **(Figure 2C)**.

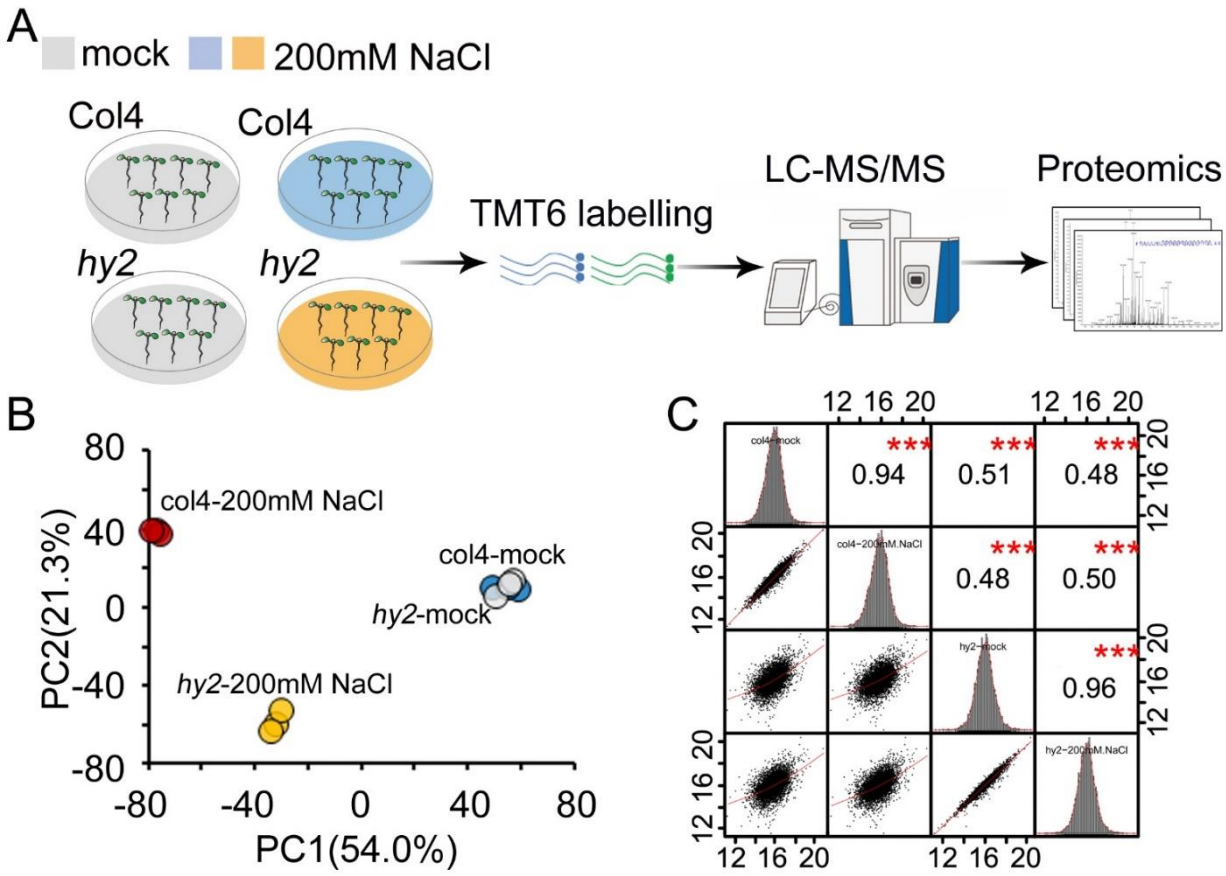


Figure 2. Quantitative proteomics analysis of col4 and *hy2* mutant under salt stress. (A) Workflow for proteomics analysis. 5 d-old col4 and *hy2* mutant seedlings were treated with or without 200 mM NaCl for 5 h. The proteomics analysis consisted of three steps. Step 1, proteins were extracted from tissues and proteolytically digested. Step 2, TMT6-labelling. Step 3, Nano-HPLC-MS/MS. **(B)** Unsupervised Principal Component Analysis (PCA) of quantitative proteomics data. **(C)** Scatterplot matrices by which linear and non-linear relations were investigated. The value represents the pearson correlation between treatments. The red asterisk represents significant difference between treatments.

2.3. Function analysis of accurate protein quantification

Among the 7,391 proteins accurately quantified, 5,995 were quantified both in col4 and *hy2* mutant, 637 were specifically quantified in col4 and 759 in *hy2* mutant **(Figure 3A, Table S2)**. Proteins with fold change ratio > 1.5 and *P* value < 0.05 were defined as DRPs, among which 31 (12 up-regulated and 19 down-regulated) were specifically responded in col4, and 19 (19 down-regulated) in *hy2* mutant **(Figure 3B)**. It was obvious that the specific DRPs quantified in *hy2* mutant were far less than those quantified in col4, indicating a correspondence to the NaCl insensitivity of *hy2* mutant. For the function of these quantified proteins specifically regulated by HY2 under NaCl stress, we carried out GO enrichment analysis, the result of which indicated that the specific DRPs in col4 were the major players of ion homeostasis and flavonoid biosynthetic & metabolic process to responde to salt stress **(Figure 3C, Table S5)** , while the specific DRPs in *hy2* mutant were mainly involved in hormone response pathway (JA,SA,ABA and ethylene), cellular detoxification and ROS metabolic pathway to responde to salt stress **(Figure 3D, Table S6)**.

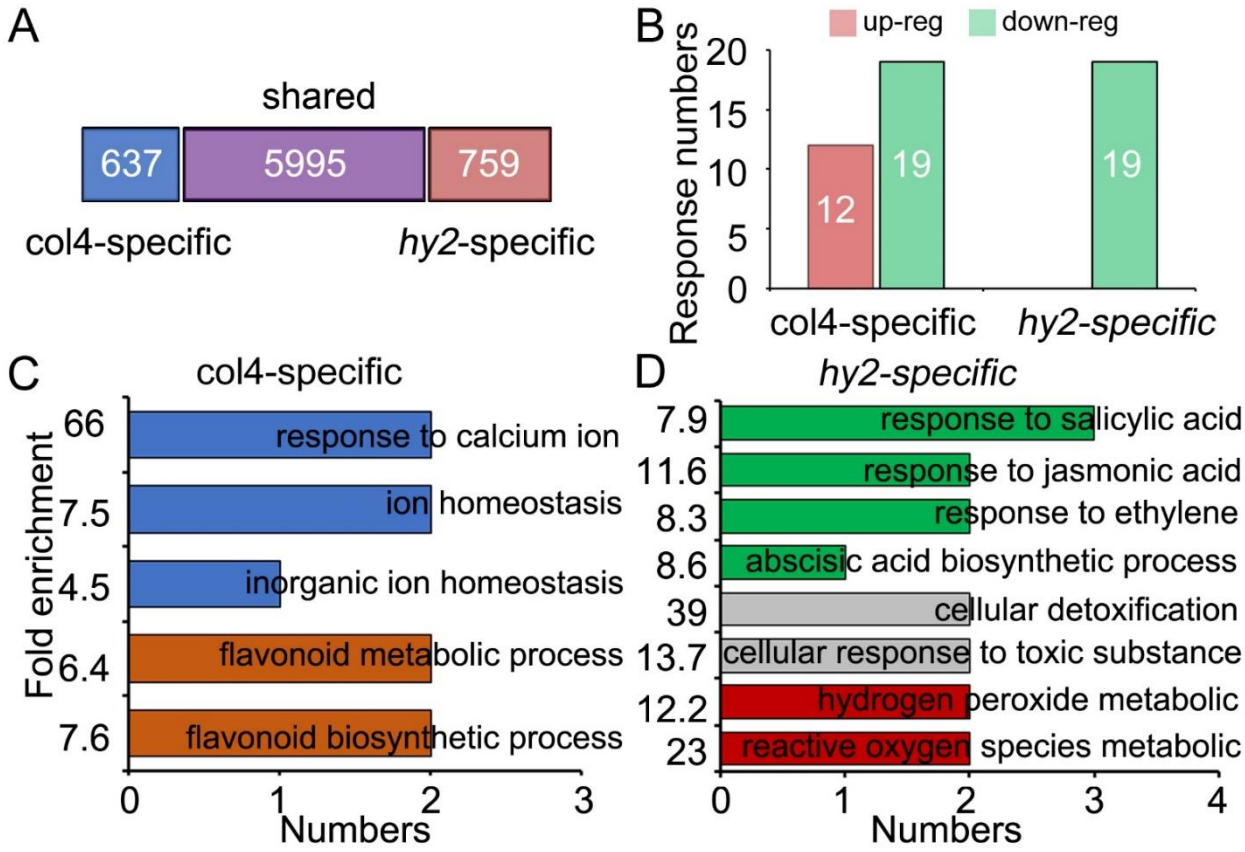


Figure 3. Function analysis of specific quantified proteins. (A) The number of specific and shared quantified proteins between col4 and *hy2* mutant under salt stress. **(B)** The response number of col4-specific and *hy2*-specific under salt stress. The red and green squares represent up-regulated and down-regulated proteins, respectively. **(C)** The GO enrichment analysis of col4-specific quantified and response proteins. **(D)** The GO enrichment analysis of *hy2*-specific quantified and response proteins.

2.4. Function analysis of CRPs

The 5,995 quantified proteins identified both in col4 and *hy2* mutant presented different protein expression patterns **(Figure 4A)**. Among these shared quantified proteins, 194 DRPs were quantified in col4, and 97 DRPs were quantified in *hy2* mutant **(Table S3,S4)**, the result of which showed that DRPs quantified in *hy2* mutant was significantly less than those in col4 (only about 50% of col4 responsive proteins), corresponding to the salt-resistant phenotype of *hy2* mutant again **(Figure S3C)**. Inside of those DRPs quantified in col4 and those in *hy2* mutant, we identified 63 shared DRPs (63 down-regulated), 131 col4-specific DRPs (84 up-regulated and 47 down-regulated) and 34 *hy2*-specific DRPs (5 up-regulated and 29 down-regulated) (**Figure 4B**). We then classified these DRPs into two groups: one was the shared DRPs in col4 and *hy2* mutant, which responded to NaCl stress but were not specifically regulated by HY2; the other were col4-specific or *hy2*-specific DRPs, which were specifically regulated by HY2. We next conducted GO analysis to study the various functions of those proteins and found that the proteins regulated by NaCl stress but not specifically regulated by HY2 mainly took part in salt stress response, stress & stimulus response, cell wall organization, ROS metabolic, detoxification and lipid transport **(Figure S3D, Table S9)**. We also found that, responding to salt stress, the proteins specifically regulated by HY2 were

mainly involved in oxidative stress response, ion transmembrane transport, photosynthesis, response to water and detoxification in col4 **(Figure 4C, Table S7)**, while those in *hy2* mutant were involved in defense response, biotic stimulus response, proteolysis and the response to wounding. **(Figure 4D, Table S8)**.

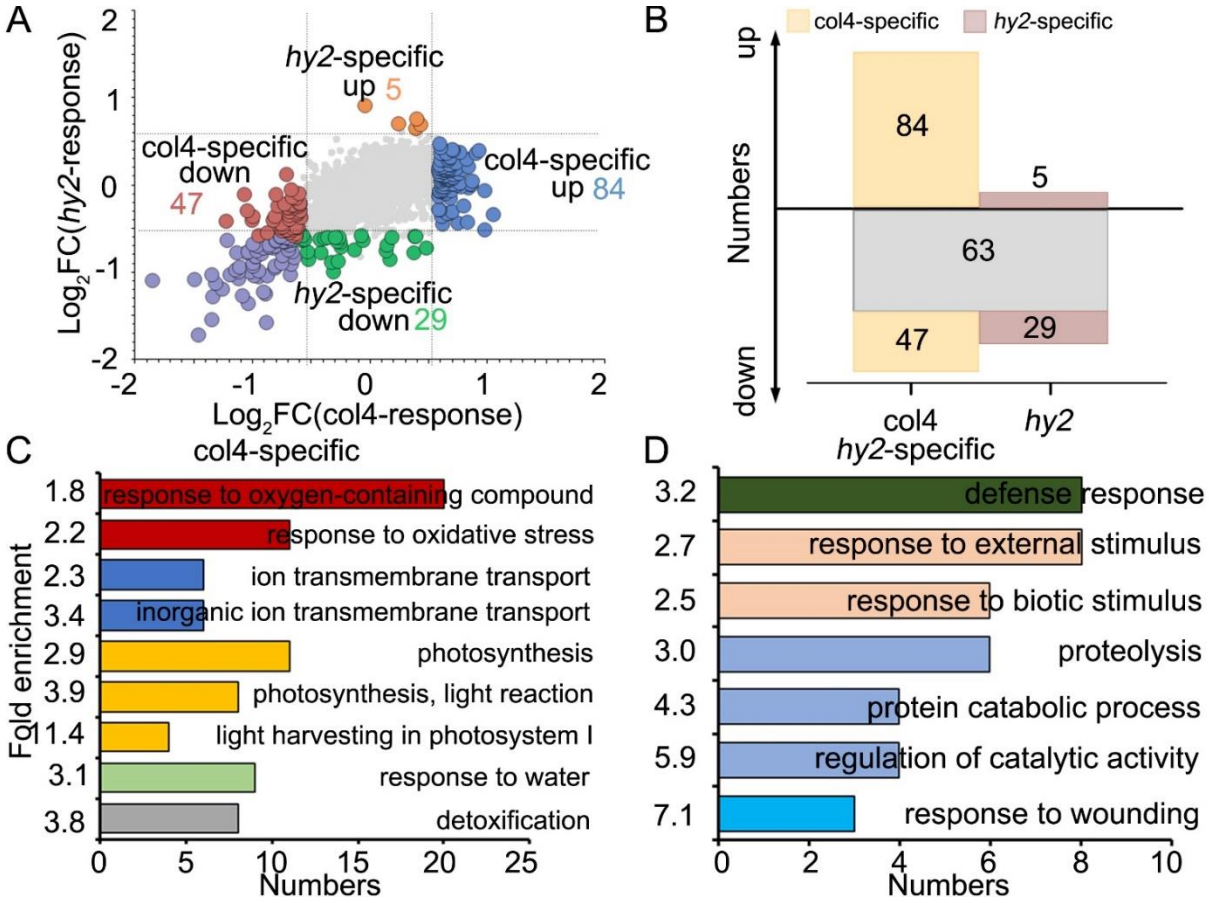


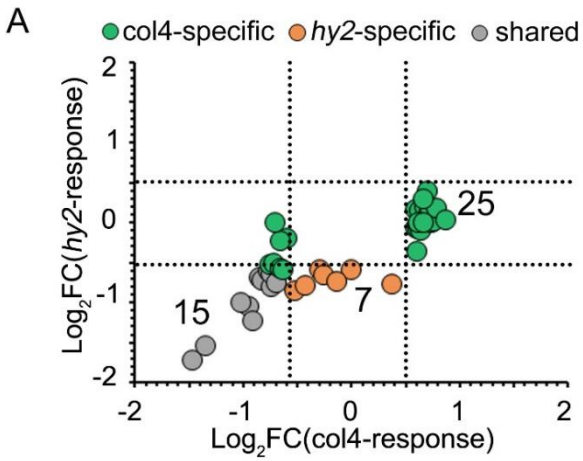
Figure 4. Function analysis of DRPs. (A) Protein expression pattern of col4 and *hy2*-shared quantified proteins. The abscissa and ordinate represent the protein pattern of col4 and *hy2* mutant, respectively. Color denotesproteins that are regulated similarly by col4 and *hy2* (grey), or specifically by col4 (blue and red) or *hy2* (orange and green). **(B)** The number of distinct proteins significantly up- or down-regulating col4 and *hy2* mutant. Color denotesproteins that are regulated similarly by col4 and *hy2* (grey), or specifically by col4(orange) or *hy2* (brown). **(C)** The GO enrichment analysis of col4-specific response proteins. **(D)** The GO enrichment analysis of *hy2*-specific response proteins.

2.5. Disruption of *HY2*-altered expression of a set of stress-responsive genes

NaCl stress is one of the stress factors that affects the growth and development of seeds, and induces the expression of stress-related proteins such as seed growth & development-related proteins and ABA pathway-related proteins. Therefore, we verified the expression patterns of proteins related to salt stress, seed growth and development, and ABA pathway in col4 and *hy2* mutant **(Figure 5A)**, from which we found 15 proteins were mediated by NaCl stress but not by HY2 in a specific way (gray dot), while 32 were specifically mediated by HY2 (green and yellow dot). All of the proteins were then divided into different groups: proteins involved in the salt stress, proteins participating in ABA pathway and proteins taking part in the growth & development of seeds, among which the number of proteins specifically regulated by HY2 (col4-specific and *hy2*-

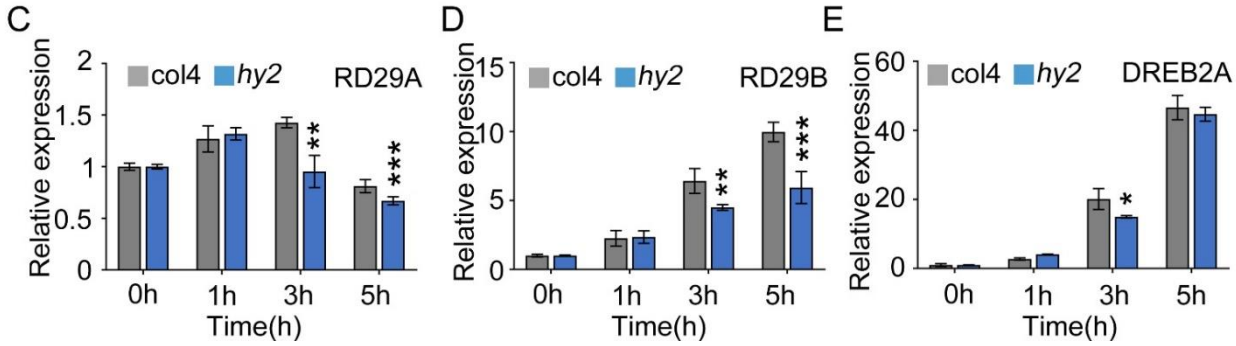
specific) were 22, 10 and 5, respectively, while the number of proteins specifically regulated by NaCl stress were only 9, 7 and 1 (less than half the number of proteins specifically regulated by HY2), respectively. These results showed, under NaCl stress conditions, that proteins involved in salt stress pathway dominated, accounting for 66%, and those involved in ABA pathway and seed growth/development took the secondary place **(Figure 5B)**, indicating that HY2 simultaneously regulates the protein expressions related to salt stress, ABA pathway and seed growth & development. As *HY2* is a potential regulator involved in NaCl signaling **(Figure 1A)**, the expression of stress inductive genes, such as *RD29A*, *RD29B* and *DREB2A*, were tested in *hy2* mutant. We tested the inducible genes under the conditions of 0 h-5 h stress of 200mM NaCl, and found that the expression levels of *RD29A* and *RD29B* were undifferentiated at 0 h and 1h in *hy2* mutant, but significantly decreased at 3 h and 5 h, when compared with those in col4 at the same conditions **(Figure 5C,5D)**; the expression levels of *DREB2A* remained the same at 0 h, 1 h and 5 h, and significantly down-regulated just at 3 h **(Figure 5E)**, indicating that *HY2* induces the the expression of NaCl inducible genes and positively regulates NaCl signaling.

Figure 5. The expression of stress-responsive genes. (A) Protein expression pattern of col4 and *hy2* shared proteins, which



B

Type \ response	Salt	ABA	Seed
shared	9 (60%)	7 (47%)	1 (6%)
col4-specific	17 (68%)	8 (32%)	4 (16%)
hy2-specific	5 (71%)	2 (29%)	1 (14%)



are involved in salt, ABA and seed related pathway. Color denotesproteins that are regulated similarly by col4 and *hy2* (grey), or specifically by col4 (green) or *hy2* (orange). **(B)** The number and proportion of col4 and *hy2* shared proteins, which are involved in salt, ABA and seed related pathway. **(C-E)** The expression of *RD29A*,*RD29B* and *DREB2A* in col4 and *hy2* mutant seedlings treated with exogenous NaCl. The 5 d-old col4 and *hy2* mutant seedlings were transferred to 1/2 MS solution with or without 200 mM NaCl for 0-5 h, and then the seedlings were harvested for QPCR. Data are shown as mean ± SD (n = 3). Asterisks indicate statistically significant differences compared with relevant col4 plants: *p<0.05, **p < 0.01, ***p < 0.001.

2.6. Interaction network of HY2-specific DRPs

Protein interaction networks were generated to evaluate the interaction of the DRPs (known and unknown proteins) specifically regulated by HY2 **(Figure 6)**. We selected 22 proteins specifically regulated by HY2 under salt stress, among which 17 were col4-specific (14 up-regulated and 3 down-regulated) and 5 *hy2*-specific (5 down-regulated). The results showed that ANNAT1-ANNAT2-ANNAT3-ANNAT4, the family members of Annexins (a family of calcium dependent membrane binding proteins), were involved in salt-stress responses specifically regulated by HY2 in a mutual-functioning way. The score of association between ANNAT1 and ANNAT2 was 0.915, which was the highest, while that between ANNAT1 and ANNAT4 was 0.805, which was the lowest. Glutathione transferase GSTU19 induced by drought, oxidative stress, and hormonal responses, Glutathione S-transferase PHI 10 (GSTF10) involved in water deprivation and toxin catabolic process, 60S ribosomal protein L5 (RPL5A,RPL5B) responsible for the synthesis of proteins in the cell as the component of the ribosome and ribosomal proteins of L7Ae/L30e/S12e/Gadd45 family protein (AT2G32060) involved in translation interactionally participated in salt-stress responses specifically regulated by HY2. The association score between AT2G32060 and RPL5A/RPL5B was 0.999 while that between GSTF10 and RPL5A/RPL5B was 0.596. We therefore identified two interaction protein networks specifically regulated by HY2 under salt stress.

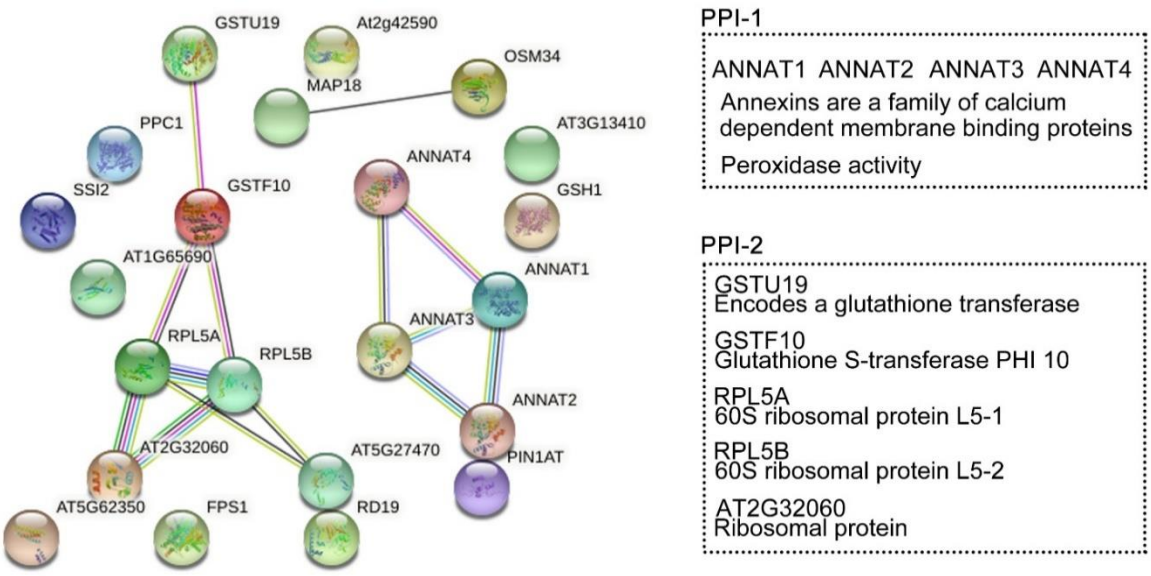


Figure 6. Protein-protein interaction(PPI) networks. STRING analysis using the HY2-specific regulated proteins under salt stress. Network nodes represent proteins. Edges represent protein-protein associations. Known Interactions: from curated databases. experimentally determined. Predicted Interactions: protein neighborhood. protein fusions. protein co-occurrence. Others: textmining. co-expression. protein homology. Tow PPI specific regulated by HY2. PPI-1:ANNAT1-ANNAT2-ANNAT3-ANNAT4. PPI-2: GSTU19-GSTF10-RPL5A-RPL5B-AT2G32060.

3. Discussion

As an increasingly serious abiotic stress factor, salt stress induced by saline soil threatens the growth and development of plants. Therefore, it is of great importance to explore related regulatory factors of salt stress and improve related pathways of salt stress [1-3]. In the current study, we found that *Arabidopsis HY2* regulates seed germination and seedling growth under salt stress as РΦВ

synthase [25,26]. We also found that *HY2* is a positive regulator to regulate the expression of downstream related genes with the physiological phenotype and biochemical analysis, and identified key proteins specifically regulated by HY2 and correlated pathways with proteomics.

Phytochrome is an important photoreceptor for plants to sense environmental changes [32-34]. *Arabidopsis hy1* and *hy2* mutants cannot synthesize photo-activated hotoactive phytochrome due to the lack of PΦB biosynthesis, resulting in impaired photomorphogenesis [26,28]. In addition, *HY2* participates in the regulation of hypocotyl elongation under far-red light, the induction of exogenous trehalose and the biosynthesis of chlorophyll [28-30], but whether it is involved in the stress response pathway is unknown. Previous studies have shown that 150-250 mM NaCl stress has serious effects on plant growth and development [35-37], therefor we used 200 mM NaCl stress as the salt stress screening condition. We next used the luciferase reporting system [38,39] to obtain a large number of LUC-tag, in which *HY2*, a positive regulator of salt stress, was screened out. The phenotype of *HY2* was then observed and the germination rate under 3-5 d NaCl stress was recorded, as a result of which we found that the best time to observe the phenotype is 4 d treatment of NaCl stress. The 3 d treatment and 5 d treatment is either early or late for such observation.

To identify the proteins and pathways specifically regulated by HY2 under salt stress, we conducted a TMT6-labeled proteomics analysis, and accurately quantified the changing patterns of 7,931 proteins under salt stress. Interestingly, we found that proteins specifically regulated by HY2 could mediate flavonoid biosynthetic process (RNS1, UGT72E1), hormone response pathway (NHL6, HR4, GAMMA-VPE) and photosynthesis pathway (PSI-P, GUN5, LHB1B1, CAB1, Lhca6, LHCA1, ATPC1, NdhL, PPC1, DUF1995 and PPC2), apart from their regulation on ion homeostasis, cellular detoxification and reactive oxygen species metabolc. According to previous studies, with salt stress, the expression patterns of 214 flavonoid biosynthetic genes in soybean changes [40], the content of flavonoid in *Solanum nigrum* raises with the increase of salt concentration [41], 584 genes in *Elaeagnus angustifolia* L. are identified and involved in photosynthetic pathways [42]. Besides, a variety of phytohormones including ABA, GA, auxin and CK are involved in the regulation of salt stress response in plants [43]. These results suggest that *HY* may be involved in the interaction between salt stress pathway and many other pathways like flavonoid pathway, plant hormone pathway, and photosynthesis pathway.

Earlier studies have shown that the expression levels of important downstream genes of *RD29A*, *RD29B* and *DREB2A* are induced by various abiotic stresses, including drought, chilling stress and salt stress [44-47]. In this study, the expression levels of downstream genes were all induced to up regulate under 0-5 h salt stress, which is consistent with previous studies. While, in *hy2* mutant, the up-regulated expression of downstream genes was inhibited, corresponding to the role of *HY2* as a positive regulator of salt stress. We used interaction network analysis and identified two interaction networks specifically regulated by HY2 under salt stress, which are ANNAT1-ANNAT2-ANNAT3-ANNAT4 and GSTU19-GSTF10-RPL5A-RPL5B-AT2G32060. Previous studies have shown that ANNAT1-ANNAT2-ANNAT3-ANNAT4, as an important membrane binding protein, participates in drought, salt stress, chilling stress and other abiotic stresses [48-51]; RPL5A-RPL5B, a 60S ribosomal protein, is involved in cold and water-deficit stresses [52,53]. These two candidate salt stress networks provide an important theoretical basis for the study of

HY2's participation in salt stress, but its specific mechanism needs further experimental verification. That how *HY2* gene accurately acts on salt stress response pathway is still an open question.

4. Materials and Methods

4.1. Plasmid construction.

Plasmids used in this study were generated by In-Fusion cloning [54,55] (https://www.takarabio.com/products/cloning/in-fusion-cloning). PEGAD-LUC vectors were used to create overexpression transgenic lines. The LUC fragment was cloned into PEGAD-MYC [56,57] to generate PEGAD-LUC vectors. The coding sequences (CDS) of *HY2* were amplified from *Arabidopsis* cDNA made previously by PCR, and the purified PCR products were then subcloned into Ecor I/Hind III-digested PEGAD-LUC vectors through In-fusion cloning.

4.2. Plant materials and growth conditions.

The wild type plant used in this study was *Arabidopsis* col4. T-DNA insertion line *hy2* mutant (SALK_104923C) was obtained from *Arabidopsis* Biological Resource Center (https://abrc.osu.edu/) and identified by the *HY2* specific primers and T-DNA left board primers. Transgenic *Arabidopsis* expressing the LUC fusion protein (LUC-HY2) was prepared by floral dipping method [58] in col4 background. LUC positive lines were screened with glufosinate, CCD camera and western blot with anti-Luciferase antibody (Sigma,L2164). Col4, *LUC-vector*, *hy2* and *LUC-HY2* were grown in Petri dishes in half-strength Murashige and Skoog salts (1/2 MS; Sigma), 1% (w/v) sucrose (Sigma), and 0.8% (w/v) agar (Sigma) in continual illumination (120 umol m^{-2} s^{-1}) at 22°C, unless specifically indicated [13]. Seeds were sown on 1/2 MS media, placed at 4°C for 3 days in the dark, and then transferred to growth rooms. The primers used for genotyping *HY2*-overexpressing lines and *hy2* mutant are listed in **Supplemental Table S10**.

4.3. Salt sensitivity assay.

Sterilized seeds were sown on 1/2 MS medium (as mock) or 1/2 MS medium containing indicated concentration of 200 mM NaCl at pH 5.8 with 0.8% (w/v) agar. After 3-5 d, seedlings were photographed, and the germination rate was determined as a percentage of total number of seeds plated. Germination was defined as an obvious emergence of the radicle through the seed coat [59]. At least 64 seedlings were observed per line, and each experiment was repeated three times.

4.4. LUC activity recordings.

LUC-HY2 overexpression lines were sown in the 96-well plate containing 1/2 MS supplemented with 0.2% (w/v) sugar, 0.4% (w/v) agar, 200 mM NaCl and 1 mM D-Luciferin (potassium Salt), with 10 seeds per well for each individual line. Seedlings were transferred to darkness for LUC activity direction [60]. LUC signals were detected in every 10 minutes, with a detecting period of 5 hours.

4.5. QPCR assay.

The total RNA of seedlings was extracted with RNeasy Plant Mini kit (QIAGEN). The total RNA (2 μg) was treated with DNase I (Takara) to eliminate genomic DNA contamination. Then the cDNA was synthesized by SuperScript II Reverse Transcriptase (Invitrogen) using Radom Hexamer Primers (Promega), then performed with 1 μl of template cDNA, 1 μl of forward primer (0.2 μm), 1 μl of reverse primer (0.2 μm), and 10 μl TB Green Premix Ex Taq in a total reaction volume of 20 μl, successively. qRT-PCR was eventually carried out to a Mx3005P Real-Time PCR

System [61,62]. The qPCR signals were normalized to that of the reference gene *actin1* using the ΔCT method [63]. There were three replicates in each sample. The primers are listed in **Supplemental Table S10**.

4.6. Tandem mass tags (TMT)-based proteomics analysis.

In order to compare the proteomics of col4 and *hy2* mutant, 7 d-old seedlings were treated with 200 mM NaCl or water (as mock) for 5 h. Approximately 0.5 g of seedlings were extracted with protein lysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% SDS, 8M Urea, 20 mM Dithiothreitol, 1x EDTA-free Protease Inhibitor Cocktail Tablets). Protein was digested using a filter-aided sample preparation (FASP) method [64]. Digested peptides were dried using a CentriVap (Thermo Fisher) and pre-fractionated with Ultimate 3000 (Thermo Fisher scientific, Waltham, MA, USA) [65]. Peptide was analyzed by on-line nanospray LC-MS/MS on an Orbitrap Fusion coupled to an EASY-nano-LC system (Thermo Scientific, MA, USA) [66,67]. All MS/MS raw data were analyzed using Proteome Discoverer 2.1 (Thermo Fisher Scientific, San Jose, CA, USA; version 2.1) [68] and Scaffold Q+ (version Scaffold_4.7.1, Proteome Software Inc., Portland, OR) [69].

4.7. GO Enrichment Analysis.

As per GO vocabulary, the sequences were characterized by OMICSBOX (www.biobam.com/omicsbox) to predict the role of contigs in biological functions (BP, MF and CC). GO enrichment analysis of DRPs were carried out to determine their roles in BP, MF and CC through OMICSBOX [70].

4.8. Interaction network analysis.

Protein-protein interaction (PPI) networks of HY2-specifically regulated proteins under salt stress were built using STRING v11 with a confidence score threshold of 0.9 (https://www.string-db.org/) [71,72].

4.9. Quantification and statistical analysis.

All statistical data were collected in a GraphPad Prism 8.0.2, ANOVA with two-tailed Student's-t test [73] was used to evaluate statistical significance, while $^{ns}p>0.05$,*$p<0.05$, **$p < 0.01$, ***$p < 0.001$. All data were reported as mean ± SD.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1-S3, Table S1-S10.

Author Contributions: M.P., J.Z., D.Y., Z.Y., X.D., Z.Z. conceived the study, designed the experiments. J.Z., Z.L., J.Z., Y.L., L.Z. performed the experiments. L.Y., N.Y., Y.L., H.T. participated in liquid chromatography-mass spectrometry (LC-MS) analysis. J.Z. analysed data. J.Z. wrote the manuscript. D.Y., Z.Y., X.D., Z.Z. critically commented and revised it. All authors have read and agreed to the published version of the manuscript.

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